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ORIGINAL ARTICLE

Liquid chromatographic and spectrophotometric methods for the determination of erythromycin stearate and trimethoprim in tablets

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Trimethoprim

Abstract Simple, accurate and precise reversed-phase liquid chromatographic (LC) and spectrophotometric methods have been developed and validated for the determination of erythromycin stearate (ERS) and trimethoprim (TMP) in mixture. In LC method, chromatographic separation was achieved on a Symmetry® Waters C18 column (150 × 4.6 mm, 5 μm) based on isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer pH (9):acetonitrile:water (25:100:50, *v*/*v*/*v*) at a flow rate of 1.6 ml min^{-1} with UV detection at 210 nm for ERS and 280 nm for TMP. Besides, two spectrophotometric methods were applied after reaction with perchloric acid (12 M) which gives a colored product with ERS. Then, the spectral interference between the colored product of ERS and TMP was resolved by either ratio spectra derivative spectrophotometry in the first spectrophotometric method or chemometric techniques, namely classical least-squares (CLS), principal component regression (PCR) and partial least-squares regression (PLS) in the second spectrophotometric method. The results were statistically compared using one-way analysis of variance (ANOVA). The methods developed were satisfactorily applied to the analysis of the

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pharmaceutical preparation containing the two drugs and proved to be specific and accurate for the quality control of the cited drugs in pharmaceutical dosage forms.

1. Introduction

Erythromycin (ER), (3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-4-[(2,6dideoxy-3-C-methyl-3-*O*-methyl-a-L-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13trihydroxy-3,5,7,9,11,13-hexamethyl-6-[(3,4,6-trideoxy-3-dimethylamino-β-D-xylo-hexopyranosyl)-oxy]oxacyclotetradecane-2,10-dione (Fig. 1a) belongs to the macrolide group of antibiotics while trimethoprim (TMP), 5-(3,4,5-trimethoxybenzyl) pyrimidine-2,4-diamine (Fig. 1b) is a dihydrofolate reductase inhibitor. The two drugs have been used in combination as antibacterial compounds.

On literature survey, erythromycin salts and esters have been determined by several methods including spectrophotometry,[1–4] spectrofluorometry,[5,6] near-infrared spectroscopy,[7] high performance liquid chromatography (HPLC).[8–19] On the other hand, trimethoprim has been determined by spectrophotometry[20–24] and high performance liquid chromatography (HPLC).[25,26] Only one article describing the analysis of ERS and TMP was found but the mixture was not analyzed simultaneously. The determination of TMP in the presence of ERS was accomplished by using zero-crossing first derivative (1D), classical least-squares regression (CLS), and principal component regression (PCR) methods. ERS was determined in the presence of TMP using 2,4-dinitrophenylhydrazine.[4] So, it was effort-worthy to develop analytical methods capable of determining the two drugs simultaneously.

LC represents an increasing growth that makes it the most popular method used in pharmaceutical analysis. HPLC greatly reduces the analysis time and allows for the determination of many individual components in a mixture using one single procedure.[27] So, it was an intention of this work to apply the LC technique for the determination of ERS and TMP in laboratory prepared mixtures and pharmaceutical preparation containing them.

Besides, due to the ease and availability of different spectrophotometric instrumentation in pharmaceutical analysis, spectrophotometry continues to be very popular, because of its simplicity and low cost so it has long been applied for the analysis of many drugs.[28–37] So, it was intended to apply spectrophotometry to the simultaneous determination of ERS and TMP. ERS reacts with perchloric acid (12 M) to give a colored product which could be measured at its maximum (482 nm) without any interference from TMP. On the other hand, TMP does not react with perchloric acid but its spectrum suffers from an overlap form the spectrum of the colored product of ERS. So, it was important to develop a method or more to solve this spectral overlap. In the first method, the determination of TMP is based on the use of the first derivative of the ratio spectra (DR_1) to eliminate any spectral interference from the colored product of ERS.

The second spectrophotometric method comprised the application of three chemometric techniques namely; classical least-squares (CLS), inverse least-squares (ILS), principal component regression (PCR) and partial least-squares (PLS) to solve spectral interference between TMP and the colored product of ERS after addition of perchloric acid. The main advantages of these techniques are the higher speed of processing data concerning the values of concentrations and absorbances of compounds with strongly overlapping spectra. Besides, the errors of calibration model are minimized by measuring the absorbance values at many points in the wavelength range of the zero-order and derivative spectra.

2. Experimental

2.1. Instrumentation

The HPLC (Merck Hitachi) instrument was used; interface D-7000 equipped with a L-7110 isocratic pump, UV–visible L-7420 detector, a manual injector equipped with (20 µl) injector loop and a Xterra C18, 250 × 4.6 mm i.d., 5 µm (Waters, Ireland) maintained at ambient temperature. Ultrasonic processor; GE 130 Fuse size 1.6 A SLO-BLO was used for degassing of the mobile phase.

The Ultraviolet/visible spectrophotometer (Shimadzu UV-1650 PC, Tokyo, Japan) connected to an IBM compatible

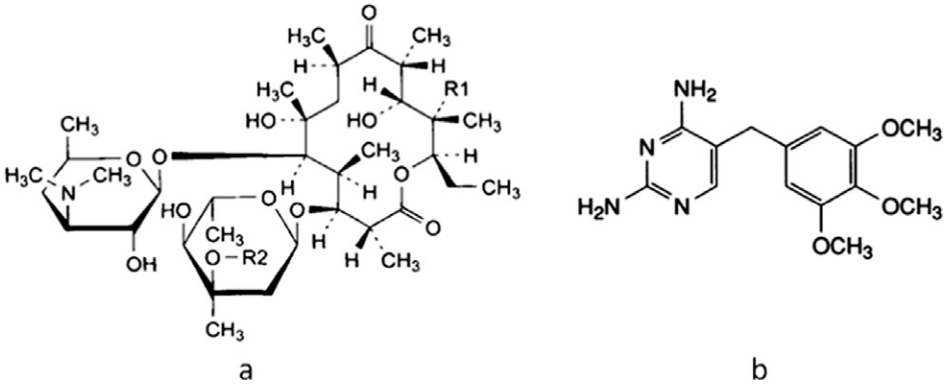

Figure 1 Chemical structure of erythromycin (a) and trimethoprim (b).

computer and supported with UVPC soft ware version 2.21 (Shimadzu Corporation, Kyoto, Japan). For the chemometric method, data analysis was performed using PLS-Toolbox 2.0 running under Matlab™, Version 7.

2.2. Reagents and reference samples

Pharmaceutical grade erythromycin stearate containing 99.80% (by microbiological assay),[38] trimethoprim containing 99.70% (by non-aqueous titration),[39] Erythroprim tablets (batch number 162077 for the chromatographic method and batch number 164115 for the two spectrophotometric methods) nominally containing ERS (equivalent to 250 mg erythromycin) and TMP (80 mg), were supplied by Misr Company for Pharmaceutical Industries, Cairo, Egypt. Methanol (HiPerSolv for HPLC), acetonitrile (HiPerSolv), potassium dihydrogen phosphate (AnalaR) and orthophosphric Acid (85%, AnalaR) were obtained from VWR Chemicals (Pool, England). Bi-distilled water was produced in-house (Aquatron Water Still, A4000D, UK). Membrane filters 0.45 μm from Teknokroma (Barcelona, Spain) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise. Standard stock solutions of each drug (1 mg ml^{-1}) were prepared by dissolving 100 mg of the drug in methanol and completing the volume to 100 ml in a volumetric flask and then the required concentrations were prepared by serial dilution.

For the LC part, liberation of erythromycin base (ER) from ERS was carried out to avoid its liberation in any part of the chromatographic system by the mobile phase. Liberation of the free base was carried out using the U.S.P method[40] as follows: an accurately weighed 694 mg ERS (equivalent to 500 mg base) was dissolved in 15 ml methanol, then 65 ml dipotassium acid phosphate buffer (pH 8) was added. The solution was filtered through a filter paper into a 100 ml volumetric flask, the residue and filter paper were washed with methanol (3 × 5 ml) and the extracts were completed to volume with methanol (5 mg ml^{-1}) and then the required concentrations were prepared by serial dilution.

2.3. Chromatographic conditions

Chromatographic separation was achieved on a Symmetry® Waters C18 column (150 × 4.6 mm, 5 μm) based on isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer pH (9):acetonitrile:water (25:100:50, *v*/*v*/*v*) with UV detection at 210 nm for ERS and 280 nm for TMP. The buffer solution was filtered through 0.45 μm membrane filter and degassed for 30 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 1.6 ml min^{-1}. Analyses were performed at ambient temperature and the injection volume was 20 μl.

2.4. Sample preparation

2.4.1. For HPLC method

Twenty tablets were weighed and finely powdered. A quantity of the powdered tablets equivalent to (500 mg) ER and (160) mg TMP was extracted and prepared as shown under Section 2.2.

2.4.2. For the spectrophotometric methods (I and II)

A quantity of the powdered tablets equivalent to (38 mg) ERS and (8.76 mg) TMP was extracted with methanol (3 × 20 ml) and filtered through a filter paper into a 100 ml volumetric flask, the residue and filter paper were washed with methanol (3 × 10 ml) and the extracts were completed to volume with methanol.

2.5. General procedures and calibration graphs

2.5.1. For HPLC method

2.5.1.1. Preparation of calibration curves. Accurately measured aliquots of erythromycin stock solution equivalent to erythromycin (10–30 mg) were transferred into a series of 10 ml volumetric flasks and the volumes were completed with methanol. For TMP, accurately measured aliquots of TMP stock solution equivalent to (3.2–9.6 mg) were transferred into a series of 10 ml volumetric flasks and the volumes were completed with methanol. Twenty microliters of each dilution was injected and the chromatograms were recorded using the chromatographic conditions mentioned in Section (2.3). Calibration curve representing areas under the peaks versus concentrations in mg ml^{-1} was constructed.

2.5.1.2. Determination of laboratory prepared mixture. Aliquots from ER and TMP stock solutions equivalent to ER (12.0–19.5 mg) and TMP (3.52–6.40 mg) were transferred into a series of 10 ml volumetric flasks and completed to volume with methanol then 20 μl of each solution were injected using the chromatographic conditions mentioned in Section (2.3).

2.5.1.3. HPLC determination of ERS and TMP in Erythroprim tablets. A quantity of the powdered tablets equivalent to (500 mg) ER and (160) mg TMP was extracted and prepared as shown under Section 2.2. Aliquots of this solution equivalent to (10.0–15.0 mg) ER and (3.20–4.80 mg) TMP were introduced into a series of 10 ml volumetric flasks and the volumes were completed with methanol. The procedure was completed as under determination of laboratory prepared mixture (Section 2.5.1.2.). The experiment was repeated applying the standard addition technique.

2.5.2. For spectrophotometric method I

2.5.2.1. Preparation of calibration curves. Different aliquots containing (0.15–0.90 mg) and (0.072–0.360 mg) of ERS and TMP stock solutions, respectively were introduced into two separate sets of 10 ml volumetric flasks followed by addition of perchloric acid (12 M) solution (3 ml). The reaction was allowed to proceed for 10 min then the volumes were completed with methanol. For ERS, The absorbance was measured at 482 nm against reagent blank. The absorbance values were plotted against concentration. For TMP, the stored spectrum of TMP was divided by the spectrum of ERS colored product (60 μg ml^{-1}) to obtain the ratio spectra then the first derivative of ratio spectra (DR_1) were obtained using the following instrumental parameters [$\Delta\lambda$ = 5 nm, scaling factor = 1 and wavelength range (200–600 nm)]. The amplitude measurements of the trough at 240 nm were plotted against concentration.

2.5.2.2. Determination of the laboratory prepared mixture. The previous procedure mentioned was applied to determine ERS and TMP in laboratory prepared mixture using aliquots equivalent to (0.35–0.75 mg) and (0.096–0.192 mg) of ERS stock

solution and TMP stock solution, respectively to check the reproducibility and repeatability of the method.

2.5.2.3. Spectrophotometric method I for the determination of ERS and TMP in "Erythroprim" tablets. A quantity of the powdered tablets equivalent to (38 mg) ERS and (8.76 mg) TMP was extracted with methanol (3×20 ml) and filtered through a filter paper into a 100 ml volumetric flask, the residue and filter paper were washed with methanol (3×10 ml) and the extracts were completed to volume with methanol. Aliquots of this solution equivalent to (0.532 and 0.570 mg) erythromycin stearate and (0.12264 and 0.1314 mg) trimethoprim were introduced into two series of 10 ml volumetric flasks. The experiment was repeated applying the standard addition technique.

2.5.3. For spectrophotometric method II

2.5.3.1. Construction of the training set. Nine binary mixtures of ERS and TMP were prepared by placing different volumes of their stock solutions into a series of 10 ml measuring flasks. Then, perchloric acid (12 M) solution (3 ml) was added to all flasks. The reaction mixture was left for 10 min and the volumes were completed with methanol. The absorbances of these mixtures were measured between 200 and 600 nm at 0.5 nm intervals against corresponding reagent blank.

2.5.3.2. Pre-processing the data. Reject the regions from 200 to 230 nm and above 505 nm.

2.5.3.3. Constructing the models. For the three techniques, the absorbance data matrix for the training set concentration matrix (Table 1) were obtained by the measurement of absorbances between 230.0 and 505.0 nm in the intervals with $\Delta\lambda = 0.5$ nm. In these techniques, calibration or regression was obtained by using the absorbance data matrix and concentration data matrix for prediction of the unknown concentrations of ERS and TMP in their binary mixtures and pharmaceutical formulations. For CLS method, CLS model was constructed with non-zero intercept. To build the CLS model, the computer was fed with the absorbance and concentration matrices for the training set. The calculations to obtain the K matrix were carried out. For the PCR and PLS models, the training set absorbance and concentration matrices together with PLS-toolbox 2.0 software were used for calculations.

Table 1 The concentrations of different mixtures of erythromycin stearate and trimethoprim used in the training set.

Sample No.	Erythromycin stearate conc. ($\mu g\,ml^{-1}$)	Trimethoprim conc. ($\mu g\,ml^{-1}$)
1	30	36
2	40	33.6
3	45	12
4	45	31.2
5	60	36
6	75	16.8
7	90	12
8	90	16.8
9	90	31.2

2.5.3.4. Selection of the optimum number of factors to build the PCR and PLS models. The cross validation method was used, leaving out one sample at a time, to select the optimum number of factors. Given a set of nine calibration samples, PCR and PLS calibrations were performed, and using this calibration, the concentration of the sample left out was predicted. The predicted concentrations were then compared with the actual concentrations and the root mean square error of cross validation (RMSECV) was calculated. The maximum number of factors used to calculate the optimum RMSECV was selected to be six. Visual inspection was used for selecting the optimum number of factors. It indicates both of the precision and accuracy of predictions. It was recalculated upon addition of each new factor to the PLS and PCR models.

$$\mathrm{RMSECV} = \sqrt{\frac{\mathrm{PRESS}}{n}}$$

where PRESS is the predicted residual error sum of squares and n is the number of calibration samples.

$$\mathrm{PRESS} = \sum \left(Y_{\mathrm{pred}} - Y_{\mathrm{true}}\right)^2$$

where Y_{pred} and Y_{true} are predicted and true concentrations in $\mu g\,ml^{-1}$, respectively.

2.5.3.5. Construction of the validation set. Different six mixtures of ERS and TMP were prepared by transferring different volumes of their stock solutions into 10 ml volumetric flasks and the procedure under "construction of the training set" was repeated. The suggested models were applied to these mixtures to predict the concentrations of ERS and TMP.

2.5.3.6. Application of the proposed chemometric techniques for the analysis of ERS and TMP in Erythroprim tablets. Aliquots of the solution prepared in section [5.2.2.3.] equivalent to (532 and 570 μg) ERS and (122.64 and 131.4 μg) TMP were introduced into a series of 10 ml volumetric flasks and the procedure under "construction of the training set" was repeated. The spectra of the prepared solutions were scanned then the developed multivariate models, CLS, PCR and PLS were applied to calculate the concentrations of ERS and TMP. The experiment was repeated applying the standard addition technique.

3. Results and discussion

3.1. HPLC method

For the separation of the examined drugs, various reversed-phase columns, isocratic mobile phase systems were attempted. Different ratios of the aqueous and organic phases were tried. The final mobile phase consisting of water, acetonitrile, dipotassium acid phosphate buffer (pH 9) in a ratio of (50:100:25, *v*/*v*/*v*) provided good separation and good peak symmetry with a steady base line. Incomplete resolution of peaks or long elution time was observed upon using ratios other than that prescribed. Acceptable retention times of the two drugs were achieved on using a flow rate 1.6 ml min^{-1}. Detection was carried out at 210 and 260 nm for ERS and TMP, respectively, at which high detector sensitivity was achieved. The retention times were 4.71 and 2.67 min for ERS and TMP, respectively as presented in Fig. 2.

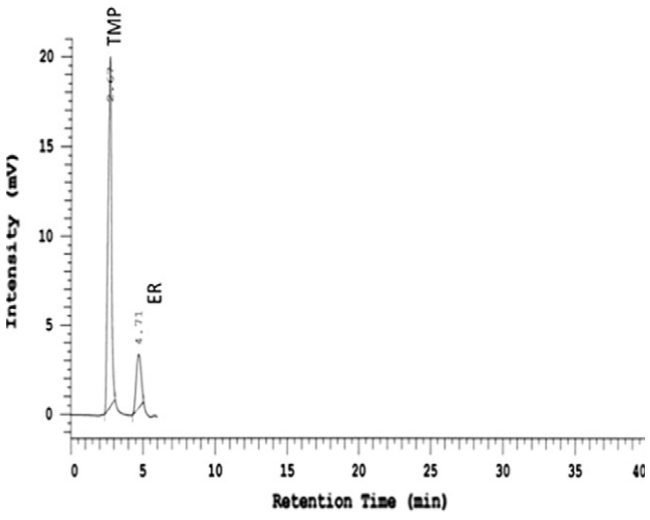

Figure 2 A typical chromatogram of a mixture of erythromycin (ER) and trimethoprim (TMP) using water: acetonitrile: dipotassium acid phosphate buffer (pH 9) (50:100:25, *v*/*v*/*v*) as mobile phase at a flow rate of 1.6 ml min^{-1}.

Table 2 System suitability tests for the proposed HPLC method for the simultaneous determination of erythromycin and trimethoprim.

Item	Erythromycin	Trimethoprim
N	1123.83	1649.02
R	4.95	
K′	3.71	1.67
R.S.D% of peak areas of six injections	1.85	1.72

3.1.1. System suitability tests

According to USP 2007,[40] system suitability tests are an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed method. System suitability tests are used to verify that resolution and reproducibility were adequate for analysis performed. Different parameters affecting the chromatographic separation were studied. The parameters of these tests are column efficiency (number of theoretical plates), tailing of chromatographic peak, peak resolution factor, and repeatability as %R.S.D of peak area for six injections and reproducibility of retention as %R.S.D of retention time. The results of these tests and their acceptance criteria according to USP regulation are listed in Table 2.

3.1.2. Linearity

Linearity was studied for ERS and TMP in binary mixture by the LC and spectrophotometric method I. A linear relationship between response studied (area under the peak (AUP) and component concentration (C) was obtained. The regression equation for each drug was also computed. In this study, five concentrations each was repeated three times. The repeated runs were genuine and not just repetition for the same readings. This approach will provide information on the variation in the peak area values between samples of the same concentration. The linearity of the calibration curves was validated by the high value of correlation coefficient. The analytical data of the calibration curves including standard deviations for the slope and intercept (S_b, S_a) are summarized in Table 3.

3.1.3. Accuracy

Accuracy of the results was calculated by % recovery of five different concentrations of the laboratory prepared mixture of the two drugs analyzed by the proposed methods and also by standard addition technique for tablets. The results obtained including the mean of the recovery, standard deviation, relative standard deviation are displayed in Table 3.

3.1.4. Precision

The repeatability of HPLC–UV detection method was assessed by analyzing a mixture containing ERS and TMP ($n = 6$). The values of the precision (%R.S.D), inter-day and intra-day precision (using three different concentrations in triplicates for three days) are displayed in Table 3.

Table 3 Results obtained by the proposed HPLC method for the simultaneous determination of erythromycin and trimethoprim.

Item	Erythromycin	Trimethoprim
Retention time	4.71 min	2.67 min
Wavelength of detection	210 nm	280 nm
Linearity range	1.0–3.0 mg ml^{-1}	0.32–0.96 mg ml^{-1}
Regression equation	$AUP \times 10^{-4} = 3.6395 \times conc_{erythromycin}$ (mg ml^{-1}) − 1.3270	$AUP \times 10^{-4} = 54.0733 \times conc_{trimethoprim}$ (mg ml^{-1}) − 2.3174
Regression coefficient (r^2)	0.9938	0.9969
S_b	0.166	1.745
S_a	0.351	1.185
Confidence limit of the slope	3.6395 ± 0.528	54.0733 ± 5.549
Confidence limit of the intercept	−1.3270 ± 1.116	−2.3174 ± 3.768
Standard error of the estimation	0.262	0.883
LOD	0.238	0.054
LOQ	0.720	0.163
Intra-day %RSD	0.74–1.02	0.63–1.11
Inter-day %RSD	1.42–2.51	0.48–1.09
Results:	99.59 ± 0.81	99.74 ± 0.86
(1) Drug in laboratory prepared mixture		
(2) Drug in dosage form	100.59 ± 0.70	94.36 ± 0.65
(3) Drug added	100.59 ± 0.65	99.29 ± 0.98

3.1.5. Selectivity

Selectivity is the ability of the analytical method to measure the analyte response in the presence of interferences including related substances. Selectivity was checked by analyzing ERS and TMP in laboratory prepared binary mixtures. Good resolution and absence of interference between drugs analyzed is shown in Fig. 2. Moreover, the proposed methods were applied to a pharmaceutical formulation containing the two drugs under study. Moreover, the chromatograms of the samples were checked for the appearance of any extra peaks. No chromatographic interference from any of the excipients was found at the retention times of the examined drugs. These results demonstrate that there was no interference from other materials in the pharmaceutical formulations and therefore confirm the specificity of the method.

3.1.6. Limit of detection and limit of quantification

Limit of detection (LOD) which represents the concentration of analyte at S/N ratio of 3 and limit of quantification (LOQ) at which S/N is 10 were determined for the proposed methods and results are given in Table 3. LOD and LOQ were computed LOD and LOQ were computed based on the standard deviation of the response and the slope.

3.2. For spectrophotometric methods I and II

3.2.1. Spectrophotometric method I

In the UV absorption spectra of ERS and TMP in methanol at their nominal concentrations in tablets, ERS spectrum is completely masked by the spectrum of strongly absorbing TMP (Fig. 3). So, direct simultaneous determination of the two drugs in mixture is not feasible. ERS, a weekly absorbing macrolide antibiotic, reacts with perchloric acid to give a colored product which could be measured at its maximum (482 nm). In literature, phosphoric acid was used for the determination of four macrolide antibiotics including erythromycin.[3] Being a stronger acid, perchloric acid was used in the present work for the analysis of ERS. ERS could be evaluated by measuring the absorbance at 482 nm without any interference from TMP.

Optimization of the reaction conditions between ERS and perchloric acid were studied. An amount of 3 ml 12 M perchloric acid was found optimum for the reaction. The reaction was allowed to proceed for 10 min and was found to be stable for 90 min. On the other hand, TMP does not react with perchloric acid but its spectrum suffers from an overlap form the spectrum of the colored product of ERS. So the determination of TMP is based on the use of the first derivative of the ratio spectra (DR_1) to eliminate any spectral interference from the colored product of ERS (Fig. 4).

According to the theory of the ratio-spectra derivative method,[29,30] the absorption spectrum of TMP was divided by a standard spectrum of ERS product. Then, the 1st derivative of the ratio-spectra were recorded and the values of the derivatives were measured at suitably selected wavelengths. Two troughs at 240 and 303 nm were obtained (Fig. 4). Measurements were carried out at 240 nm as it gave more reproducible results for TMP.

An accurate choice of either standard divisors or working wavelengths is fundamental for the application of derivative ratio method. In particular, by increasing or decreasing the concentration of divisor, the resulting derivative values and, hence, the slope of lines of regression are proportionately decreased or increased, with consequent variation of both sensitivity and linearity range. Several tests were made in a preliminary investigation by using standard divisors in the concentration range from 50 to 80 μg ml^{-1}. The best results in terms of sensitivity, repeatability were found by using 60 μg ml^{-1} of ERS colored product spectrum as the chosen divisor. The method was reproducible in laboratory prepared

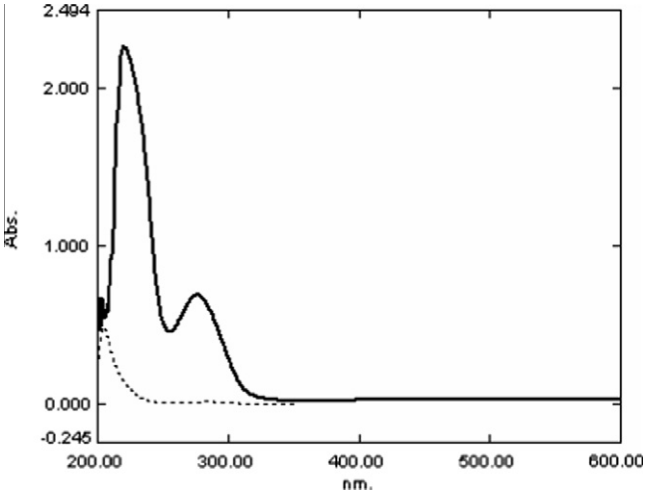

Figure 3 Zero order absorption spectra of erythromycin (0.5 mg/10 ml) (– – – – –) and of trimethoprim (0.168 mg/10 ml) (—) in methanol.

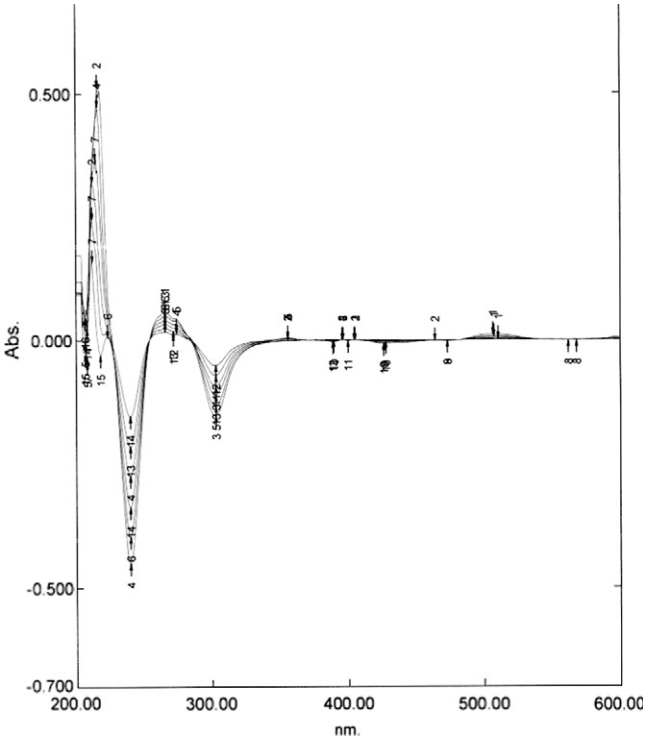

Figure 4 The first derivative ratio spectra (DR_1) of trimethoprim (12–36 μg ml^{-1}) (divisor: 60 μg ml^{-1} erythromycin stearate coloured product).

Table 4 Results obtained by the proposed spectrophotometric method I for the determination of erythromycin stearate and trimethoprim.

Item	Erythromycin stearate	Trimethoprim
λ_{max} of measurements	482 nm	240 nm
Obedience of Beer's law	15–90 μg ml^{-1}	7.2–36 μg ml^{-1}
Regression equation	$A_{482\ nm} = 0.012 \times conc_{erythromycin\ stearate}$ (μg ml^{-1}) + 0.1076	$DR_{1trimethoprim} = 0.0143 \times conc_{trimethoprim}$ (μg ml^{-1}) + 0.0028
Regression coefficient (r^2)	0.9968	0.9997
S_b	0.0003	0.0001
S_a	0.017	0.003
Confidence limit of the slope	0.012 ± 0.0007	0.0143 ± 0.0003
Confidence limit of the intercept	0.1076 ± 0.042	0.0028 ± 0.008
Standard error of the estimation	0.019	0.003
Results	99.87 ± 0.87	100.17 ± 0.89
(1) Drug in laboratory prepared mixture		
(2) Drug in dosage form	103.43 ± 0.2	97.01 ± 0.06
(3) Drug added	99.87 ± 0.76	99.76 ± 0.74

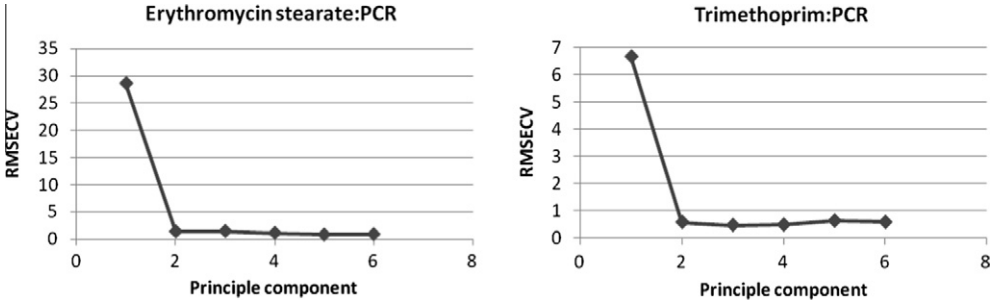

Figure 5 RMSECV plot of a calibration set prediction using cross validation (principal component regression model).

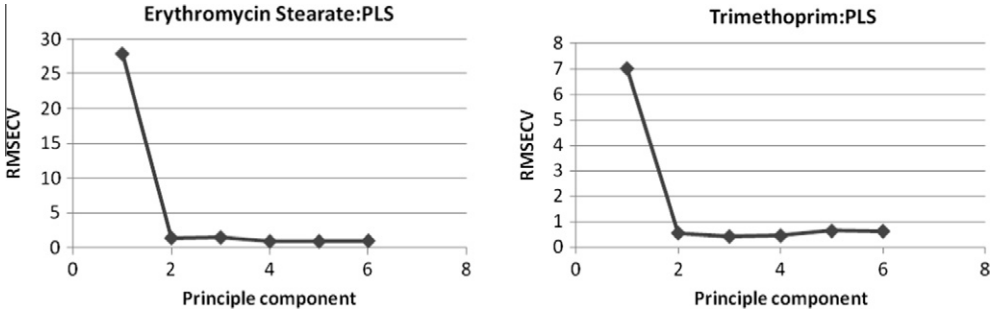

Figure 6 RMSECV plot of a calibration set prediction using cross validation (partial least squares model).

Table 5 Results obtained by the proposed spectrophotometric method II for the determination of erythromycin stearate and trimethoprim.

Item	Erythromycin stearate			Trimethoprim		
	CLS	PCR	PLS	CLS	PCR	PLS
(1) Drug in laboratory prepared mixture	99.31	100.07	100.07	99.56	99.43	99.43
(2) Drug in dosage form	103.43	103.98	103.56	96.15	96.17	96.22
(3) Drug added	99.32	99.99	100.37	100.20	100.52	100.54

mixtures and erythroprim tablets. Besides, good results were obtained for the standard addition indicating good accuracy of the method (Table 4).

3.2.2. Spectrophotometric method II

The wavelength range 230–505 nm in the intervals with 0.5 nm intervals was chosen as it was providing the greatest amount of

Table 6 Tests of significance for the proposed HPLC method for the simultaneous determination of erythromycin and trimethoprim.

Statistical term	Erythromycin		Trimethoprim	
	Reference method**	HPLC method	Reference method***	HPLC method
Mean	100.52	99.59	99.51	99.74
±S.D.	1.44	0.81	1.99	0.86
±S.E.	0.64	0.36	0.89	0.38
%RSD	1.43	0.81	2.00	0.86
n	5	5	5	5
V	2.07	0.66	3.96	0.74
t (*2.306)		1.267		0.238
F (*6.39)		3.136		5.350

* Figures in parentheses are the theoretical *t* and *F* values at ($p = 0.05$).
** B.P. 1998 (microbiological assay).[38]
*** B.P. 2007 (non-aqueous titration).[39]

Table 7 Tests of significance for the proposed spectrophotometric method for the determination of erythromycin stearate and trimethoprim.

Statistical term	Erythromycin stearate		Trimethoprim	
	Reference method**	Spectrophoto-metric method	Reference method***	Spectrophoto-metric method
Mean	100.52	99.87	99.51	100.17
±S.D.	1.44	0.87	1.99	0.89
±S.E.	0.64	0.39	0.89	0.40
%RSD	1.43	0.87	2.00	0.89
n	5	5	5	5
V	2.07	0.76	3.96	0.79
t (*2.306)		0.867		0.676
F (*6.39)		2.724		5.013

* Figures in parentheses are the theoretical *t* and *F* values at ($p = 0.05$).
** B.P. 1998 (microbiological assay).[38]
*** B.P. 2007 (non-aqueous titration).[39]

Table 8 Tests of significance for the proposed chemometric methods for the simultaneous determination of erythromycin stearate and trimethoprim.

Statistical Term	Erythromycin stearate				Trimethoprim			
	Reference method**	CLS non-zero	PCR	PLS	Reference method***	CLS non-zero	PCR	PLS
Mean	100.52	99.31	100.07	100.07	99.51	99.56	99.43	99.43
±S.D.	1.44	0.94	0.63	0.63	1.99	0.85	0.83	0.83
±S.E.	0.64	0.38	0.26	0.26	0.89	0.35	0.34	0.34
%RSD	1.43	0.95	0.63	0.63	2.00	0.85	0.83	0.83
n	5	6	6	6	5	6	6	6
V	2.07	0.88	0.40	0.40	3.96	0.72	0.69	0.69
t (*2.262)		1.626	0.652	0.652		0.052	0.084	0.084
F (*6.26)		2.352	5.175	5.175		5.500	5.739	5.739

* Figures in parentheses are the theoretical *t* and *F* values at ($p = 0.05$).
** B.P. 1998 (microbiological assay).[38]
*** B.P. 2007 (non-aqueous titration).[39]

information about the mixture components. CLS model was constructed with non-zero intercept. The non-zero intercept allows an additional degree of freedom when *K* matrix is calculated. This provides an additional opportunity to adjust the effects of the extraneous substances.[41] Selection of the optimum number of factors for the PCR and PLS techniques was a very important step before constructing the models. If the number of factors retained was more than the required, more noise will be added to the data. On the other hand, if the number retained was less than the required, meaningful data that could be necessary for the calibration might be ignored. To select the number of factors in the PLS and PCR algorithms, a cross-validation method leaving out one sample at a time[41] was employed using calibration set of 9 calibration spectra. Two factors were found suitable for both PCR and PLS methods as shown in Figs. 5 and 6.

The chemometric methods (CLS, PCR and PLS) were applied successfully to the analysis of ERS and TMP in validation set and erythroprim tablets. To assess the accuracy of the method, standard addition technique was carried out. The results were found satisfactory indicating that the additives of the tablets did not interfere. The results of validation, tablets and drug added are presented in Table 5.

3.2.3. Statistical analysis

A statistical analysis of the results obtained by the proposed methods and the reference methods was carried out by ''SPSS statistical package version 11''. The significant difference between groups was tested by one way ANOVA (F-test) at $p = 0.05$ as shown in Tables 6–8. The test ascertained that there was no significant difference among the methods.

4. Conclusion

The three proposed methods have the advantages of simplicity, precision, accuracy and convenience for the separation and quantification and can be employed for the assay of their respective dosage form. So the proposed methods can be used for the quality control of the cited drugs in ordinary laboratories.

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